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Mechanistic Insight into Pertussis Toxin and Lectin Signaling Using T Cells Engineered To Express a CD8α/CD3ζ Chimeric Receptor

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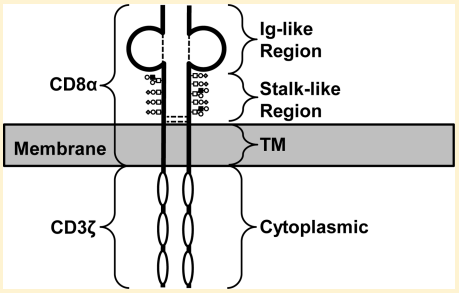
Supporting Information

ABSTRACT: Mammalian cell-surface receptors typically display N- or O-linked glycans added post-translationally. Plant lectins such as phytohemagluttinin (PHA) can activate the T cell receptor (TCR) and other cell-surface receptors by binding to glycans and initiating receptor cross-linking. Pathogenic microorganisms such as *Bordetella pertussis* also express proteins with lectin-like activities. Similar to plant lectins, pertussis toxin (PTx) can activate the TCR and bind to a variety of glycans. However, whether the lectin-like activity of PTx is responsible for its ability to activate TCR signaling has not been formally proven. Here we examined the ability of PTx and a panel of lectins to activate the TCR or a CD8α/CD3ζ chimeric receptor (termed CD8ζ). We demonstrate that CD8ζ rescues PTx-induced signaling events lacking in TCR null cells. This result indicates that CD8ζ can substitute for TCR and supports the hypothesis that PTxB (functioning as a lectin) stimulates signaling via receptor cross-linking rather than by binding to a specific epitope on the TCR. Moreover, PTx is able to activate signaling by binding either N-linked or O-linked glycan-modified receptors as the TCR displays N-linked glycans while CD8ζ displays O-linked glycans. Finally, studies with a diverse panel of lectins indicate that the signaling activity of the lectins does not always correlate with the biochemical reports of ligand preferences. Comparison of lectin signaling through TCR or CD8ζ allows us to better define the structural and functional properties of lectin−glycan interactions using a biologically based signaling readout.



Glycans are among the most abundant molecules present on the surface of cells. Cells and tissues possess unique patterns of glycosylation, which provide an additional layer of control in a variety of biological processes, including pathogen recognition, cell signaling, and leukocyte extravasation. The two main forms of protein glycosylation are termed N-linked and O-linked. In the case of N-linked glycosylation, long oligosaccharide chains containing a mannose core are added to asparagine residues; for O-linked glycosylation, short oligosaccharide chains are attached to serine or threonine residues, and mannose is usually excluded. Proteins termed "lectins" possess one or more glycan binding sites and are produced by a wide variety of organisms, including animals, plants, and bacteria.

Several lectins derived from plants, including concanavalin A (ConA), phytohemagglutinin (PHA), and wheat germ agglutinin (WGA), have been central to T cell research because of their ability to activate the T cell receptor (TCR) in a manner that is independent of antigen. These lectins can bind to multiple components of the TCR complex and induce clustering of intracellular receptor chains. This leads to phosphorylation of the immunorecptor tyronsine-based activation motifs (ITAMs) and activation of downstream signaling molecules (Figure 1). Most plant lectins contain multiple glycan binding sites, but they vary widely in structure and glycan recognition preferences. Table 1 summarizes the preferred binding ligands for a variety of lectins based on previously published data and glycan array data made publically available by the Consortium for Functional Glycomics (http://functionalglycomics.org). While ConA, PHA, and WGA can cluster and activate the TCR, they do so through interactions with different glycans. ConA is a homotetramer, and each subunit contains a binding site for α-D-mannosyl oligomers found on N-linked glycans.[1,2] PHA is a tetramer, formed from two different types of subunits, PHA leucoagglutinin (PHA-L) and PHA erythroagglutinin (PHA-E), which can associate in any combination.[3] PHA-L binds to the Galβ1−4GlcNAcβ1−2Man sequence commonly found in N-linked glycans, while PHA-E binds to terminal Gal and GlcNAc groups on branched N-linked glycans.[5,6] The PHA-L homotetramer is a strong activator of the TCR, while the PHA-E homotetramer primarily mediates red cell agglutination.[4] WGA is a homodimer, and each subunit has two glycan binding sites.[7] WGA can bind either GlcNAc or Neu5Ac present on N- or O-linked glycans.[8,9]

In addition to the plant lectins, it is now clear that pathogenic microorganisms express proteins with lectin-like activities. One example is the *Bordetella pertussis*-encoded pertussis toxin (PTx). PTx is an AB_5 toxin comprised of a hexameric polypeptide complex with five binding (B) subunits arranged in a ring structure and a single active (A) subunit with enzymatic properties sitting on top of the pore of the ring

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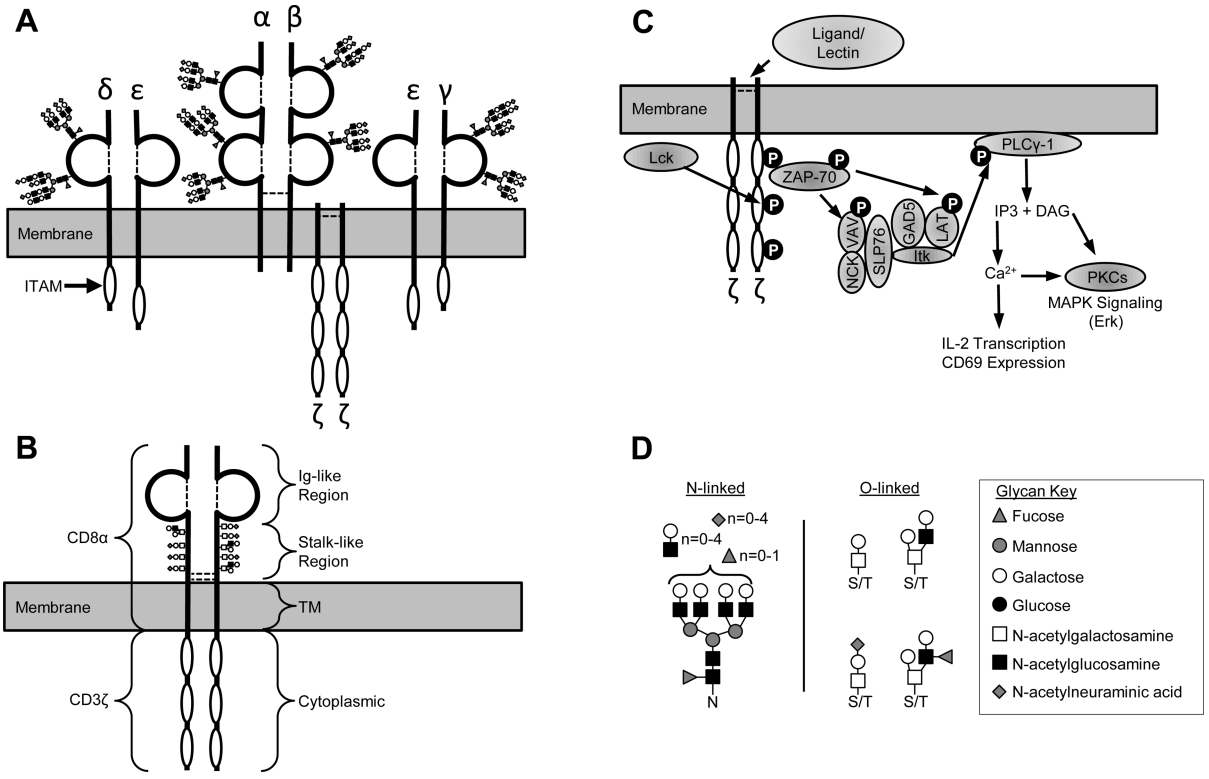

Figure 1. Schematic representation of TCR and CD8ζ structure, glycosylation patterns, and signaling ability. (A) Schematic representation of the TCR complex showing the *α*, *β*, *γ*, *δ*, *ε*, and *ζ* chains. Disulfide bonds are represented by dashed lines; immunoglobulin-like domains are represented by three-quarter circles, and cytoplasmic ITAM motifs are represented by white ovals. N-Linked glycans representative of those typically found on the TCR are shown in CFG symbol nomenclature (key in panel D). (B) Schematic representation of the CD8ζ chimeric protein (315 amino acids). Residues 1−203 are from CD8*α* and contain an Ig-like fold, a stalklike region, and a transmembrane domain. Residues 204−315 are from the cytoplasmic domain of CD3ζ, which contains three ITAM motifs. The chimera is expressed as a disulfide-linked homodimer, with extensive O-linked glycosylation in the stalklike region. (C) Schematic representation of the signaling pathway downstream of ζ chain phosphorylation that can be activated by clustering either TCR *α*/*β*-chains or CD8ζ chimeric molecules. (D) Representative examples of N-linked and O-linked glycans typically found on human T cells according to the CFG glycan profiling database. Glycans are shown in CFG symbol nomenclature. N represents asparagine and S/T serine/threonine. *n* indicates the number of modified branches.

structure. The A subunit of PTx, S1, is an ADP-ribosyltransferase that targets the *α*-subunit of some GTP-binding proteins.[10] The five B subunits of PTx (collectively termed the B pentamer or PTxB) are required for binding and cytosolic entry of S1 into mammalian cells. Unlike other AB_5 toxins, which have five identical B subunits, PTxB is comprised of four different subunits (S2−S5) in a 1:1:2:1 ratio. All the binding activities have thus far been mapped to the S2 and S3 subunits of PTxB.[11−22] Analogous to WGA, each S2 and S3 subunit contains multiple glycan binding sites. Interestingly, although the sequences of S2 and S3 are 71% identical, each has distinct binding preferences. PTx has been shown to bind a broad array of glycans, including sialylated and nonsialylated N-glycans, sialylated O-glycans, and sialylated gangliosides.[23]

The glycan binding activity of PTxB mediates activities independent of its role in delivering the S1 catalytic subunit to cellular targets. Via the B subunits, PTx can bind to a variety of cellular receptors and activate their associated signaling pathways.[24] Receptor targets for PTxB include the TCR in T cells, Toll-like receptor 4 (TLR4) in dendritic cells, and CD14 in myelomonocytic cells.[25−33] In T cells, the binding of PTxB to the TCR leads to T cell activation and mitogenesis.[24] The various receptors that PTxB bind share little structural similarity but are heavily glycosylated.[34−36] The broad binding specificity of PTx likely allows it to act as a superlectin, able to trigger signaling by clustering a wide variety of glycosylated receptors.[37−42] This hypothesis, however, has yet to be formally proven.

Studies that aim to improve our understanding of the molecular basis of lectin activity in cellular systems have been hampered by several factors. One problem is the incomplete understanding of the repertoire of binding sites for each particular lectin. Glycans also typically interact with their cognate binding sites with very low affinity, and tight binding is often achieved by engaging multiple binding sites. Additionally, because cell-surface glycans are built by sequential enzymatic processing, they can be in various stages of "completion", resulting in considerable heterogeneity. Furthermore, a single cell-surface protein may display both N-linked and O-linked glycosylation. Some of these problems have been overcome by the development of glycan arrays similar to that established by the Consortium for Functional Glycomics. These arrays can be very useful for identifying binding sites for lectins by identifying similar motifs within different glycans. However, technical issues can complicate the analysis, necessitating additional confirmatory studies. For example, if the glycans on the array are not spaced to allow for engagement of multiple binding sites or if the lectin in question binds multiple different ligands by distinct mechanisms, array-type experiments are less useful. A cellular system in which some of these variables could be experimentally controlled would contribute valuable information to our understanding of PTxB and lectin action.

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Table 1. Signaling and Binding Activity of Bacterial and Plant Lectins

lectin	abbreviation	preferred ligand[a]	protein glycan targets	Ca^{2+} signaling			ERK signaling		
				WT	CD8ζ	TCR−	WT	CD8ζ	TCR−
Erythrina cristagalli lectin	ECL	Galβ1−4GlcNAc	N-glycans, O-glycans	+	+	−	+	ND	+
Jacalin (*Artocarpus integliforia*)	JAC	Galβ1−3GalNAc (T antigen)	O-glycans	+	+	−	+	+	+
Vicia villosa agglutinin	VVA	GalNAcα1-Ser/Thr (Tn antigen)	O-glycans	+	+	−	+	+	+
Phaseolus vulgaris erythroagglutinin	PHA-E	GlcNAcβ1−2Manα1−3(GlcNAcβ1−4)(Galβ1−4GlcNAcβ1−2Manα1−6)Man	N-glycans	+	+	−	+	+	−
Bordetella pertussis toxin	PTx	NeuAc, complex-type N-glycans	N-glycans, O-glycans	+	+	−	+	+	−
wheat germ agglutinin (*Triticum aestivum*)	WGA	GlcNAc, NeuAc	N-glycans, O-glycans	+	+	−	+	+	−
succinylated wheat germ agglutinin (*T. aestivum*)	sWGA	GlcNAc	N-glycans, O-glycans	+	+	−	+	+	−
soybean agglutinin (*Glycine max*)	SBA	terminal GalNAc	N-glycans, O-glycans	+	+	−	+	+	−
Ricinus communis agglutinin	RCA	terminal Gal	N-glycans, O-glycans	+	+	−	ND	ND	ND
P. vulgaris leucoagglutinin	PHA-L	Galβ1−4GlcNAcβ1−2Man	N-glycans	+	−	−	+	+	−
Datura stramonium lectin	DSL	GlcNAcb1−4, Galb1−4GlcNAc	N-glycans, O-glycans	+	−	−	+	+	−
Lens culinaris agglutinin	LCA	branched (Manα)$_n$ with Fucα1−6GlcNAc	N-glycans	+	−	−	+	−	−
Pisum sativum agglutinin	PSA	branched (Manα)$_n$ with Fucα1−6GlcNAc	N-glycans	+	−	−	+	−	−
Concanavalin A (*Canavalia ensiformis*)	ConA	branched (Manα)$_n$	N-glycans	+	−	−	+	−	−
Griffonia simplicifolia lectin II	GSL II	terminal GlcNAc	N-glycans, O-glycans	+	−	−	−	−	−
Lycopersicon esculentum lectin	LEL	GlcNAc	N-glycans, O-glycans	+	−	−	−	−	−
Dolichos bitflorus agglutinin	DBA	GalNAcα1−3GalNAc, GalNAcα1−3(Fucα1−2)Gal (A antigen)	N-glycans, O-glycans	−	−	−	−	−	−
peanut agglutinin (*Arachis hypogaea*)	PNA	terminal Galβ1−3GalNAc (T antigen)	O-glycans	−	−	−	−	−	−
Sophora japonica agglutinin	SJA	terminal GalNAc/Gal	N-glycans, O-glycans	−	−	−	−	−	−
Ulex europaetus agglutinin	UEA-I	Fucα1−2Gal (H antigen)	N-glycans, O-glycans	−	−	−	−	−	−

[a]The stated ligand preferences are based on information available from public databases [Consortium for Functional Glycomics (http://www.functionalglycomics.org) and Lectin frontier Database (http://riodb.ibase.aist.go.jp/rcmg/glycodb/LectinSearch)] and published reports of lectin−glycan binding.[1−9,23,62−64] ND means not determined.

PTxB activates a canonical TCR/CD3 signaling pathway, including events such as phosphatidylinositol (PI) hydrolysis, Ca^{2+} flux, and MAP kinase activity.[24,43,44] Furthermore, while wild-type Jurkat cells robustly respond to PTxB, Jurkat derivatives lacking the TCR are devoid of any detectable TCR-related signaling responses to PTxB.[24] The lack of PTx-induced signaling in cells missing the TCR provides the opportunity to engineer TCR minus mutant cell lines to express exogenously added "receptor" components to rescue PTx-induced signaling. In this report, we used the system previously described by Irving et al. to engineer a TCR minus Jurkat T cell line to express a chimeric protein containing the extracellular CD8α domain and the intracellular CD3ζ domain (CD8ζ chimera).[45,46] Comparing the responses of the CD8ζ chimera to those of the wild-type TCR is a useful model system for studying the mechanism of action of molecules such as PTx, ConA, PHA, etc. Additionally, this model system can provide important insights into the structural and functional properties of TCR activation.

MATERIALS AND METHODS

Cell Lines and Reagents. The human Jurkat T cell lymphoma line (clone E6.1) was obtained from the ATCC, while the J.EMS-T3.3[47,48] Jurkat derivative lacking TCR expression was obtained from A. Weiss (University of California, San Francisco, CA). All cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 μg/mL). PTx and PTxB were purchased from List Biologicals. The presence of contaminating PTx holotoxin in PTxB preparations was assessed using the CHO cell clustering assay.[49] The concentration of residual PTx holotoxin in PTxB preparations was found to be <2%. No effect was observed in experiments using the PTx holotoxin at these concentrations in short-term assays; therefore, we conclude that the observed effects are due to PTxB and not contaminating catalytic subunit activity. PTx was inactivated by being boiled for 30 min. Stimulating antibodies, αCD8 monoclonal antibody (mAb) clone OKT8 and αCD3 mAb clone HIT3a, were purchased from eBioscience and BD Pharmingen, respectively. Antibodies used in immunoprecipitation assays, anti-ZAP-70 (clone 2F3.2) and the anti-phosphotyrosine antibody (clone 4G10),

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were obtained from Upstate Biotechnology.[50,51] Western blotting antibodies for phosphorylated and total p44/42 ERK MAP kinases were obtained from Cell Signaling Technologies. The CD8ζ chimera construct (comprised of the extracellular and transmembrane domains of CD8α fused to the cytoplasmic domain of CD3ζ) was a kind gift from A. Weiss.

Construction of a Jurkat Derivative Cell Line Expressing the CD8ζ Chimera. The CD8ζ chimera was subcloned into the *Hpa*I site of the Mig-R1 retroviral vector (Mig-R1/CD8ζ). Mig-R1 is a bicistronic retroviral vector containing a multiple-cloning site upstream of an internal ribosome entry site followed by the cDNA encoding enhanced green fluorescent protein (GFP).[52] The Mig-R1 and Mig-R1/CD8ζ constructs were cotransfected with the envelope construct encoding pantropic VSVg into GP-293 cells (HEK cells stably expressing retroviral *gag* and *pol*) (Clontech). Jurkat cells were then transduced with the HEK supernatant by spinfection at 1800 rpm for 45 min, and gene expression from Mig-R1 was monitored by FACS analysis for GFP. After several weeks in culture, the top 10% of GFP-expressing cells from each population were selected using a FACSAria cell sorter and expanded. Expression of the CD8ζ chimera was analyzed by flow cytometric analyses of live cells stained with the anti-CD8 mAb (OKT8) primary antibody and anti-mouse PE secondary antibody.

Immunoprecipitations and Western Blotting Assays. Jurkat cells (2.5×10^7) were washed once with PBS and suspended in serum-free RPMI. The samples were slowly warmed to 37 °C and then stimulated with 10 μg/mL anti-CD3 mAb, anti-CD8 mAB, or PTxB for 5 min. Samples were washed once with ice-cold PBS and lysed in lysis buffer [1% NP-40, 0.1% SDS, 0.1% deoxycholate, 150 mM NaCl, 50 mM Tris, 20 mM Na_3VO_4, and Complete protease inhibitor tablets (Roche)]. Lysates were clarified by centrifugation, and supernatants containing 1 μg of immunoprecipitating antibody per 100 μg of total protein were incubated overnight at 4 °C with 30 μL of 60% protein A/G bead slurry (Calbiochem) or combined with 3× Laemmli sample buffer for whole cell extracts. Beads were washed four times with 1 mL of lysis buffer, suspended in 3× Laemmli sample buffer, boiled for 10 min, and electrophoresed in 8% sodium dodecyl sulfate−polyacrylamide gel electrophoresis gels.[24] The gels were transferred to nitrocellulose, probed with the appropriate primary and secondary antibodies, and visualized via chemiluminescence (Super Signal, Pierce).

Measurement of ERK Activity. Cells (1×10^6 per treatment) were serum starved when indicated and stimulated for various times with 2 or 5 μg/mL PTxB, 2 μg/mL α-CD3, or 2 μg/mL anti-CD8 mAb. After the indicated treatment, cells were lysed directly in Laemmli sample buffer and analyzed for phosphorylated ERK 1 and 2 (phospho-ERK) and total ERK by Western blotting.[24]

Inositol Phosphate Assays. Jurkat cells (5×10^5 cells/mL) were labeled with 1.5 μCi/mL [^{3}H]myo-inositol (Perkin-Elmer) for 18 h in RMPI containing 10% serum. The cells were washed with cold PBS and suspended at a density of 10^7 cells/mL in serum-free RMPI containing 20 mM LiCl; 10^6 cells/treatment were stimulated with designated amounts of 5 or 10 μg/mL PTxB, 2 μg/mL anti-CD3 mAb, 2 μg/mL anti-CD8 mAb, or 10 μg/mL PHA for 2 h at 37 °C. After stimulation, the cells were lysed with 0.4 M perchloric acid at 4 °C for 15 min and neutralized with 0.72 M KOH and 0.6 M $KHCO_3$. Total inositol phosphates were isolated with Dowex resin (Bio-Rad), washed with water, and eluted with 1 M ammonium formate and 0.1 M formic acid. The eluted samples were counted with a liquid scintillation counter, and the percent conversion of inositol phosphates from total incorporated ^{3}H was calculated.[24] Data were graphed and analyzed using GraphPad Prism 4.

Ca^{2+} Flux Assays. Cells (2×10^5 cells/sample) were washed with serum-free RPMI and plated. Cells were loaded with the Fluo-4 NW Calcium Assay Kit (Invitrogen) according to the manufacturer's protocol and treated with PBS (without Ca^{2+} or Mg^{2+}), PTxB, αCD8 mAb, αCD3 mAb, or lectin (2 μg/mL). Ca^{2+} flux was analyzed[24] by the increase in fluorescence reported by the Flex Station II (Molecular Probes) using SoftMax Pro. Data were imported into GraphPad Prism 4 for analysis and production of graphs.

Aggregation Assays. Cells were washed with serum-free HBSS (Gibco) and plated in 96-well microtiter plates at a density of 1×10^6/mL. Cells were treated with HBSS, PTxB, or lectin (2 μg/mL). The microtiter plates were then incubated at 37 °C and observed for agglutination after 1−2 h.

RESULTS

Generation and Characterization of TCR Minus Jurkat Cells Stably Expressing the CD8ζ Chimera. To gain mechanistic insight into bacterial and plant lectin signaling through Ig superfamily receptors such as TCR/CD3, we sought to investigate lectin-mediated activation of a chimeric "receptor" termed CD8ζ, which contains the extracellular and transmembrane portions of human CD8α chain fused to the intracellular cytoplasmic domain of CD3ζ (Figure 1B).[45,46,53,54] Previous studies indicated that this chimera activates a classical signaling pathway similar to that activated by TCR/CD3 (Figure 1C).[45,46] Additional studies with the CD8ζ chimera demonstrated that signaling through CD3 can be propagated by clustering or aggregating the three cytoplasmic ITAM motifs found in each CD3ζ molecule, and that signaling through CD3 does not necessarily require ligand-induced conformational changes in the α/β-chains of the TCR.[45,46,55,56] Interestingly, the extracellular ligand binding domains of the TCR and CD8α differ in their glycosylation status; the TCR α/β-chains contain predominantly N-linked glycans, while CD8α contains only O-linked glycans.[35,53,57] Computational profiling of the TCR and CD8 polypeptides supports these data as the TCR α/β-chains contain eight predicted sites of N-linked glycosylation while the CD8 α-chain contains seven predicted sites of O-linked glycosylation (Table S1 of the Supporting Information).[58,59] By comparing PTx- and lectin-stimulated signaling through the TCR or the CD8ζ chimera, we can ask specific questions regarding the potential role of the peptide backbone structure and glycosylation status in receiving and propagating the PTx/lectin signal.

The CD8ζ chimeric gene was transduced into the TCR negative Jurkat T cell line (clone J.EMS-T3.3[47,48]) using a Mig-R1/CD8ζ retrovirus. Because Mig-R1/CD8ζ drives gene expression with a bicistronic mRNA simultaneously expressing GFP and CD8ζ,[52] we were able to use cell sorting based on high-level GFP expression to generate a stable CD8ζ positive cell line. Cells transduced with the empty Mig-R1 retrovirus vector (designated TCR$^-$) expressed high levels of GFP (Figure 2A) compared to untransduced controls (data not shown) but did not express any detectable CD8 or CD8ζ (Figure 2B). In contrast, cells transduced with the Mig-R1/CD8ζ retroviral vector (designated CD8ζ) expressed high

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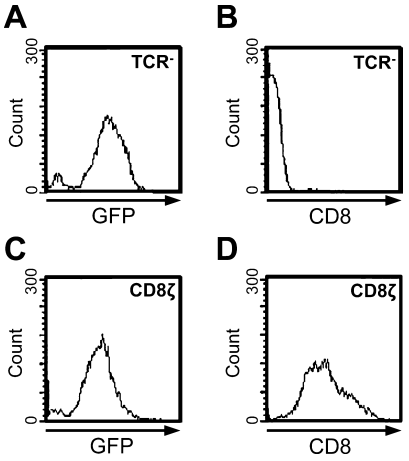

Figure 2. Retroviral expression of CD8ζ in TCR minus Jurkat T cells. TCR minus Jurkat T cells (clone J.EMS-T3.3) were transduced with the empty Mig-R1 retrovirus (A and B) or with a Mig-R1/CD8ζ retrovirus (C and D). Cells expressing a high level of GFP were sorted and reanalyzed by FACS for GFP expression (A and C) or CD8 expression (B and D).

levels of GFP (Figure 2C) and also expressed high levels of CD8ζ (Figure 2D). These cells, which express CD8ζ on a TCR null background, serve as the basis for the following studies.

CD8ζ Substitutes for the TCR and Rescues PTx Signaling in TCR⁻ Jurkat Cells. Next, we determined if expression of the CD8ζ chimera could rescue PTxB-stimulated signaling events, which occur in WT Jurkat but not in TCR minus Jurkat cells.[24] WT, TCR⁻, or CD8ζ cells were stimulated for 2 h with PTxB, anti-CD3, or anti-CD8 antibodies and analyzed for the production of inositol phosphates (InsP) (Figure 3). Consistent with previous reports,[24,43,45] WT Jurkat cells exhibited increased InsP levels when treated with the anti-CD3 antibody (Figure 3A), CD8ζ cells exhibited increased InsP levels when treated with the anti-CD8 antibody (Figure 3C), and TCR⁻ cells exhibited no detectable response to either antibody (Figure 3B). PTxB strongly induced the accumulation of InsP in WT cells (Figure 3A), but not in TCR⁻ cells (Figure 3B), and expression of CD8ζ rescued the ability of PTxB to induce InsP accumulation (Figure 3C). This result indicates that CD8ζ can substitute for the TCR and supports the hypothesis that PTxB (functioning as a lectin) stimulates signaling via receptor cross-linking and/or clustering rather than by binding to a specific epitope in the TCR α/β-chains and initiating signaling via a conformational change in the α/β-chains themselves.

PTxB-Mediated Signaling through the CD8ζ Chimera Involves Canonical TCR Signaling Components. On the basis of the observation that PTxB signaling was rescued by expression of the CD8ζ chimera, we next determined whether PTxB-activated CD8ζ signaling events were similar to those typically initiated by the TCR.[45,46,55] WT Jurkat cells stimulated with PTxB or anti-CD3 antibodies exhibit increased levels of tyrosine phosphorylation of proteins at molecular masses of 100, 70, and 35 kDa, consistent with that reported for canonical TCR signaling and observed in earlier studies by our laboratory (Figure 4A).[24] Interestingly, CD8ζ cells stimulated with PTxB or anti-CD8 antibodies demonstrated increases in tyrosine phosphorylation similar to that observed in WT cells stimulated with PTxB or anti-CD3 antibodies, indicating that the CD8ζ chimera activates a signaling pathway very similar to that of

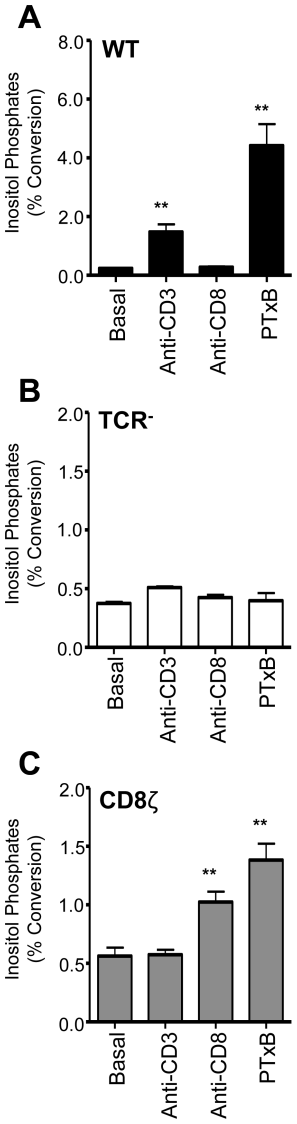

Figure 3. PTx utilizes CD8ζ to induce inositol phosphate accumulation. WT (A), TCR⁻ (B), or CD8ζ (C) Jurkat cells were left unstimulated or stimulated with 2 μg/mL anti-CD3, 2 μg/mL anti-CD8, or 10 μg/mL PTxB for 2 h, and inositol phosphate levels were determined. Statistical analysis using a Student's *t* test was performed comparing each treatment to the basal sample for each cell type (**$p < 0.01$).

TCR/CD3.[45,46,55,60−62] Increases in the level of tyrosine phosphorylation were not seen in TCR⁻ Jurkat cells following treatment with PTxB, anti-CD3, or anti-CD8 antibodies (data not shown).[24]

Phosphorylation of ZAP-70 is thought to be the most critical event in TCR activation, as it is responsible for the phosphorylation of downstream proteins, including SLP-76 and PLCγ-1.[50,60,62] To determine if ZAP-70 was tyrosine-phosphorylated and therefore active in CD8ζ cells stimulated with PTxB, we stimulated WT or CD8ζ Jurkat cells with PTxB or the appropriate control antibody, isolated ZAP-70 by immunoprecipitation, and probed Western blots with anti-phosphotyrosine or anti-ZAP-70 antibodies. WT Jurkat cells treated with an anti-CD3 antibody or PTxB exhibited increased levels of tyrosine phosphorylation of ZAP-70 (Figure 4B). Similarly, CD8ζ cells treated with the anti-CD8 antibody or PTxB displayed increased levels of tyrosine phosphorylation of

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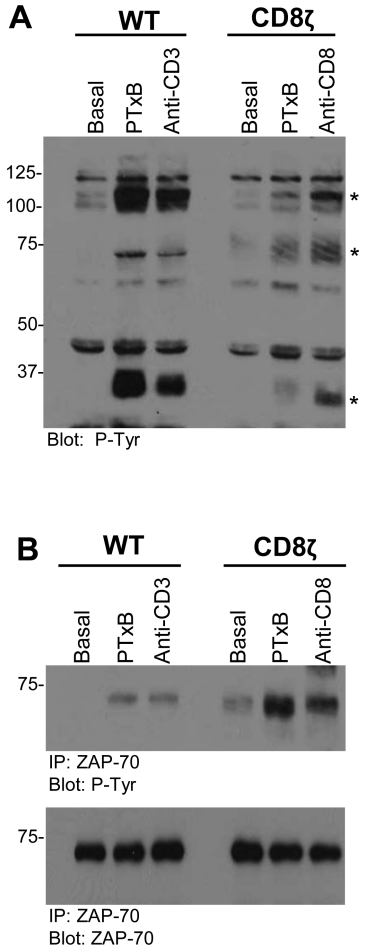

Figure 4. PTx utilizes CD8ζ to activate key TCR signaling proteins. WT or CD8ζ Jurkat cells were left untreated or stimulated with 10 μg/mL PTxB, 5 μg/mL anti-CD8 (CD8ζ cells only), or 5 μg/mL anti-CD3 (WT cells only) for 5 min. (A) Tyrosine-phosphorylated proteins were analyzed in the whole cell extracts. Proteins phosphorylated in response to PTxB are denoted with asterisks. (B) ZAP-70 was immunoprecipitated from lysates using an anti-ZAP-70 mAb. An anti-phosphotyrosine antibody (clone 4G10) was used to detect tyrosine phosphorylation. Representative blots are shown for each analysis.

ZAP-70. Thus, these results indicate that CD8ζ can substitute for TCR/CD3 to activate CD3-dependent signaling, and that PTxB can utilize either the native TCR/CD3 complex or CD8ζ to initiate the activation of canonical TCR signaling.

PTx Utilizes CD8ζ To Activate the MAP Kinase Pathway. PTxB can activate the ERK MAP kinase in endothelial cells, monocyte-like cells, and T cells.[24,26,29,31] In T cells, this activity has been attributed to the expression and function of the TCR.[24] To determine if PTxB can also use the CD8ζ chimera to drive ERK signaling, we assayed ERK phosphorylation in WT, TCR$^-$, and CD8ζ Jurkat cells. Consistent with previous findings, WT Jurkat cells treated with either anti-CD3 or PTxB displayed increased levels of phosphorylation of ERK 1/2 (Figure 5, left panel), and TCR$^-$ Jurkat cells failed to respond to any treatment (Figure 5, middle panel). CD8ζ cells treated with either anti-CD8 or PTxB displayed increased levels of phosphorylation of ERK 1/2 (Figure 5, right panel). CD8ζ cells treated with anti-CD3 antibodies did not show an increase in the level of phosphorylated ERK 1/2, confirming that the observed

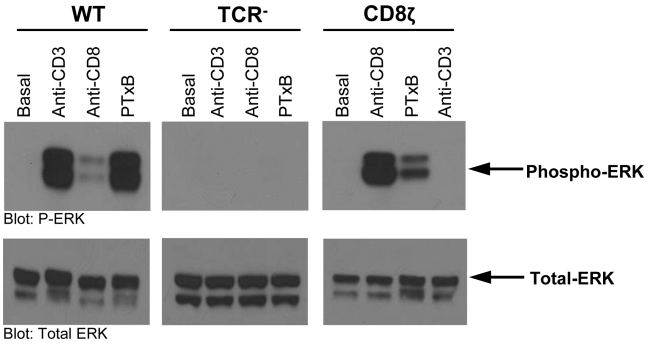

Figure 5. PTx utilizes CD8ζ to activate the MAP kinase pathway. WT (left), TCR$^-$ (middle), or CD8ζ (right) Jurkat T cells were left unstimulated or stimulated with 1 μg/mL anti-CD3, 1 μg/mL anti-CD8 mAb, or 5 μg/mL PTxB for 15 min. Whole cell extracts were analyzed by Western blotting for activated ERK with phospho-specific antibodies, or total ERK with anti-ERK antibodies. Representative blots are shown.

PTxB-induced InsP accumulation was a result of CD8ζ signaling and not low-level TCR expression (Figure 5C). Thus, as in InsP accumulation experiments described above, PTxB can also activate the ERK MAP kinase pathway in T cells using either the TCR or CD8ζ.

PTx Utilizes CD8ζ To Induce Calcium Signaling. In addition to accumulation of InsP and MAP kinase activity, PTxB has been shown to promote a rapid and prolonged elevation of the intracellular level of Ca^{2+} in T cells in a TCR-dependent manner and therefore provides a platform for confirming the effects of PTxB signaling through the CD8ζ chimera.[24,43] Ca^{2+} flux in response to anti-CD3, anti-CD8, or PTxB was examined in all three Jurkat cell derivatives (Figure 6). The anti-CD3 antibody stimulated Ca^{2+} flux only in WT cells (Figure 6A), and the anti-CD8 antibody stimulated Ca^{2+} in only the CD8ζ-expressing cells (Figure 6B). WT and CD8ζ-expressing cells stimulated with PTxB displayed a rapid and sustained calcium response that remains elevated for more than 5 min post-PTxB addition (Figure 6C), although the magnitude of the PTxB response was smaller than that observed for anti-CD3 or anti-CD8. The TCR$^-$ cells exhibited a transient spike in the intracellular level of Ca^{2+} in response to PTxB (Figure 6C). This response was nonspecific and unrelated to the biological activity of the toxin itself, as cells stimulated with 5 μg/mL boiled PTxB (Figure 6D) or 5 μg/mL BSA (data not shown) also demonstrated this rapid transient spike. Altogether, these Ca^{2+} results support our earlier studies indicating that PTxB can utilize either the TCR or the CD8ζ chimera to induce rapid signaling in T cells.

Comparison of the Ability of Plant Lectins To Signal through the TCR or CD8ζ. Many of the plant lectins are known to activate TCR signaling via the binding of glycan moieties typically found on the TCR α- and β-chains.[63] However, it remains unknown if these lectins would function like PTxB and exhibit biological activity toward other Ig superfamily members or, more specifically, would be capable of activating the CD8ζ chimera. Therefore, we compared the ability of various lectins to activate calcium signaling through the TCR (in WT Jurkat cells) or the CD8ζ chimera (in the CD8ζ stables). Both wheat germ agglutinin (WGA) and soy bean agglutinin (SBA) function like PTx in that they stimulated Ca^{2+} signaling via either TCR or CD8ζ (Figure 7A,B). This is consistent with their ability to bind either N-linked (TCR) or

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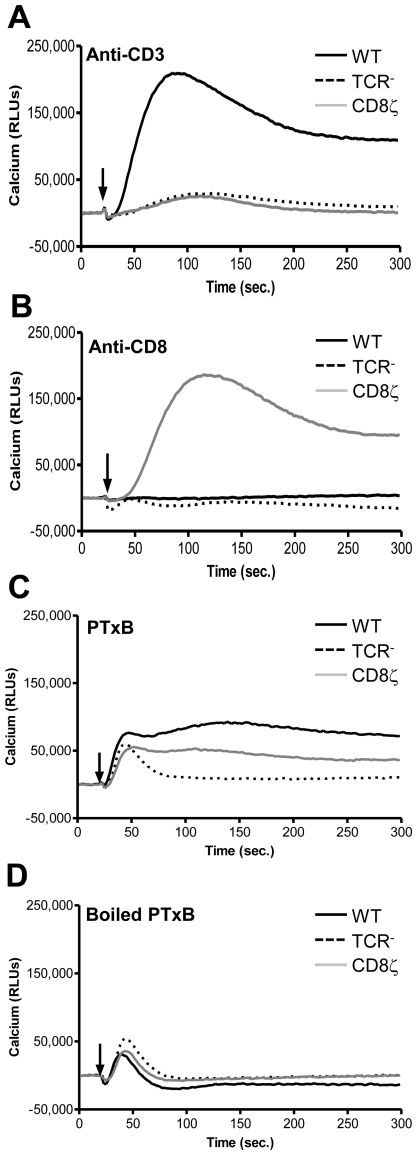

Figure 6. PTx induces calcium flux in CD8ζ Jurkat cells. WT, TCR$^-$, or CD8ζ Jurkat cells were loaded with the Fluo-4 NW calcium indicator dye and stimulated with 1 μg/mL anti-CD3 (A), 1 μg/mL anti-CD8 (B), 5 μg/mL PTxB (C), or 5 μg/mL boiled PTxB (D). The time at which ligand is added is marked with a black arrow. Data were collected on a Molecular Dynamics FlexStation. Representative data are shown for each treatment.

O-linked (CD8ζ) glycans (Table 1). Phytohemagluttinin leucoagglutinin (PHA-L) and concanavalin A (ConA) are also capable of activating Ca^{2+} signaling via the TCR, but not via CD8ζ (Figure 7C,D), which is consistent with their ability to primarily bind N-linked glycans (Table 1). In contrast, peanut agglutinin (PNA) and *Dolichos bilforous* agglutinin (DBA) are unable to activate either receptor type (Figure 7E,F).

To validate our assay using the CD8ζ chimera as a biological tool to explore aspects of lectin binding and signaling, we examined PHA-L-mediated accumulation of InsP. PHA-L is able to induce Ca^{2+} signaling through the TCR, but not through CD8ζ (Figure 7C), and therefore, we would expect PHA-L to behave similarly in InsP assays. As predicted, PHA-L is indeed capable of inducing the accumulation of InsP in WT Jurkat cells but is unable to stimulate the accumulation of InsP in CD8ζ cells (Figure 8). Because PHA-L is known to bind predominantly to complex N-glycans on the TCR[63] (Table 1 and Figure 1) but not to O-glycans on CD8, our results demonstrate that comparing lectin signaling via the TCR versus CD8ζ is a useful approach to exploring lectin−glycan interactions on a biological level. Specifically, we can differentiate between lectin responses mediated by receptors decorated with N- or O-linked glycans.

Next, we broadened our study to include a large panel of plant lectins (Table 1) that were selected to represent a diverse cross section of lectins that have the ability to bind to an equally diverse set of ligands. The preferred binding ligand denoted for each lectin in Table 1 is based on a consensus of published reports and glycoarray results, although from analyses of glycoarray results, it is clear that lectin−glycan binding cannot be simplified to state that a particular lectin can interact with only one particular ligand (Table S2 of the Supporting Information). The abilities of these lectins to activate downstream signaling events in WT, CD8ζ, or TCR$^-$ cells were assessed by monitoring Ca^{2+} responses (Table 1). The signaling responses for many lectins (WGA, sWGA, SBA, RCA, LCA, PSA, and ConA) were entirely consistent with their predicted abilities to bind N-linked or O-linked glycans. We also examined ERK activation, and the results are largely consistent with the observed Ca^{2+} responses (Table 1). However, some lectins exhibited biological responses not predicted by their binding preferences (Table 1 and Figure 9). For example, while ECL, JAC, and VVA require either the TCR or CD8ζ to induce Ca^{2+} signaling (Figure 9A−C), these lectins were able to activate ERK signaling in cells lacking the TCR or CD8ζ (Figure 9D,E). This suggests that these lectins activate a receptor(s) linked to ERK, but distinct from one that uses the canonical TCR signaling pathway involving ZAP-70, PLCγ1, etc.

Several lectins (DBA, PNA, SJA, and UEA-1) did not display any signaling activity in our assays, suggesting that neither TCR nor CD8ζ displays the appropriate glycans for these lectins, or they were unable to cross-link the receptors in a way that promotes activation. Because lectin activity is commonly associated with the ability to aggregate cells, each of the three cell types (WT, CD8ζ, and TCR$^-$) was incubated with the lectins in Table 1, and cellular aggregation was assessed (data not shown). Lectin-mediated cellular aggregation was seen in all cases where cellular signaling (Ca^{2+} and/or ERK activation) was observed. In contrast, cells treated with DBA, PNA, SJA, and UEA-1 (which failed to induce Ca^{2+} or ERK signaling) did not display any aggregation, suggesting that Jurkat cells fail to display the terminal glycan required for responsiveness to these lectins.

DISCUSSION

A CD8/CD3ζ chimeric receptor engineered to artificially link the extracellular domain of CD8α to the intracellular domain of CD3ζ has been shown to transduce signaling events typically associated with the TCR/CD3 complex when stimulated with the α-CD8 antibodies.[45,46] Unlike the TCR α/β-chains, which are modified predominantly by N-linked glycans, the CD8ζ chimera contains O-linked glycans.[35,53,57−59] This difference in glycan modification on an otherwise common signaling background allowed us to examine the ability of PTxB and a diverse panel of lectins to activate canonical CD3 signaling via receptors modified with N-linked or O-linked glycans. PTxB

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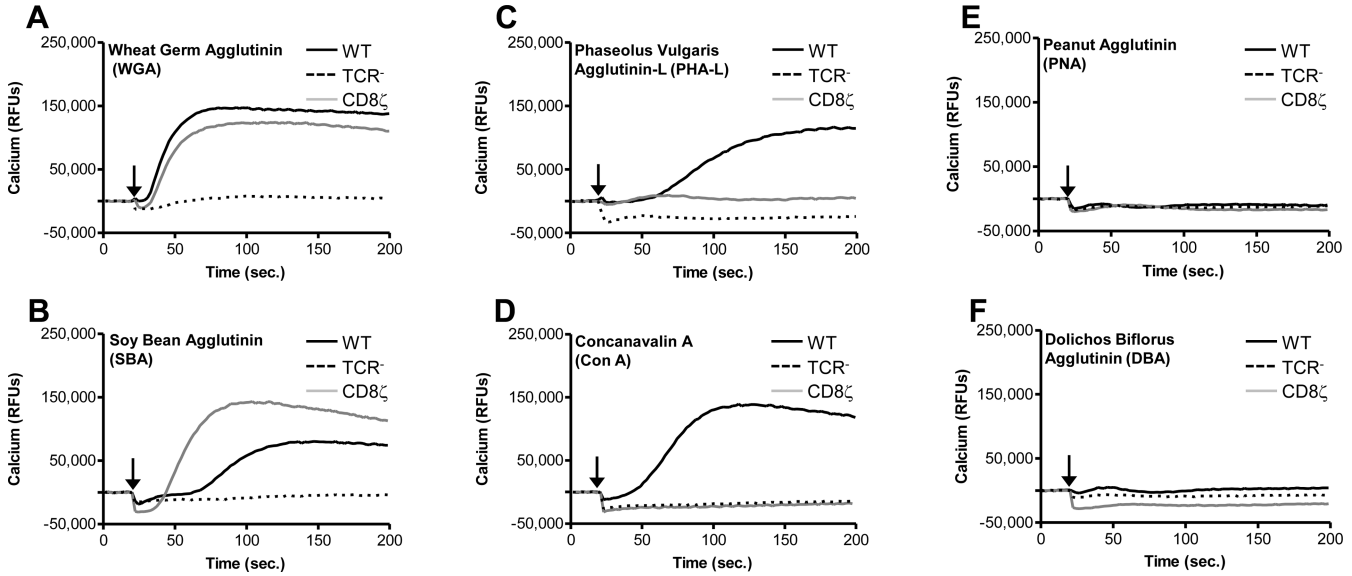

Figure 7. Lectin-mediated Ca^{2+} signaling. WT, TCR^-, or CD8ζ Jurkat cells were loaded with the Fluo-4 NW calcium indicator dye and stimulated with 2 μg/mL WGA (A), SBA (B), PHA-L (C), ConA (D), PNA (E), or DBA (F). The time at which ligand is added is marked with a black arrow. Data were collected on a Molecular Dynamics FlexStation. Representative data are shown for each treatment.

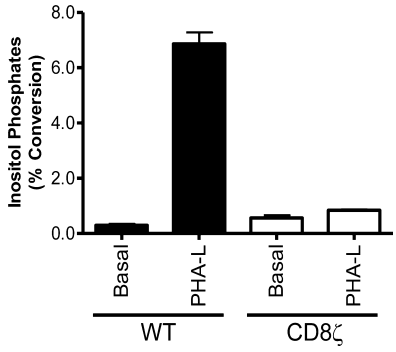

Figure 8. PHA-L utilizes the TCR but not CD8ζ to activate signaling events. WT or CD8ζ Jurkat cells were untreated or stimulated with 10 μg/mL PHA-L for 2 h, and inositol phosphates were analyzed ($n = 2$).

has at least four glycan binding sites and can bind sialylated and nonsialylated N-glycans, sialylated O-glycans, and sialylated gangliosides.[23] In previous studies,[25−32] PTxB has been shown to activate a variety of receptors, including the TCR, TLR4, and CD14. In this study we have shown that PTxB also activates the CD8ζ chimera. Specifically, the CD8ζ chimera rescued PTxB-activated signaling events in TCR^- Jurkat cells, including an increased level of tyrosine phosphorylation of TCR signaling molecules (e.g., ZAP-70), accumulation of inositol phosphates, activation of ERK, and mobilization of intracellular Ca^{2+} (Figures 3−6). Together, these results strongly support the hypothesis that PTxB does not recognize amino acid sequences present on receptor molecules but rather acts like a superlectin to promote signaling by clustering a wide variety of glycosylated receptors.

We did observe some differences in the magnitude of signaling when comparing TCR versus CD8ζ activity. While the anti-CD8 antibodies induced signaling via the CD8ζ chimera to levels comparable to that of anti-CD3 stimulation of the TCR (Figures 3−6), PTxB-mediated activation and lectin-mediated activation of signaling via these two receptors were not always equivalent. For example, PTxB activated ERK (Figure 5) and mobilized Ca^{2+} (Figure 6C) more efficiently on cells expressing wild-type TCR than on cells expressing the CD8ζ chimera, suggesting there is an inherent difference between the TCR and CD8ζ complexes that makes the TCR a better receptor for PTxB. Whether this is attributed to binding efficiency or some other factor remains unclear at this point.

We also examined the ability of various plant lectins to initiate activation of signaling activities downstream of the wild-type TCR or CD8ζ chimera, including mobilization of intracellular Ca^{2+} and activation of ERK (Figures 7−9 and Table 1). The activation patterns for many of these lectins, either via the TCR (containing N-linked glycans) or via the CD8ζ chimera (containing O-linked glycans), could be predicted on the basis of their preference for binding to N- or O-linked glycans. However, some of the lectins displayed activities in the biological assays that would not have been predicted on the basis of their glycan binding profiles in cell-free systems. For example, while PHA-E has not been previously reported to bind to O-linked glycans, it does activate signaling via the CD8ζ chimera (Table 1). The preferred ligand for PHA-E is particularly complex, requiring three different binding elements: a bisecting GlcNAc group, a Gal group at the nonreducing terminus of the Man(α1−6) branch, and a GlcNAc group on the Man(α1−3) branch.[64] In this case, the preferred ligand represents the highest-affinity ligand discovered through careful biochemical analyses, and while each of the binding elements is required for high-affinity binding, some ligands lacking an individual element still bound, albeit with reduced affinity. A branched N-linked glycan can display different sugars on each branch and can satisfy this requirement for different adjacent glycans. In contrast, O-linked glycans display minimal branching, and a single glycan cannot provide the three binding elements for PHA-E. However, as seen in Figure 1B, each CD8 monomer possesses five closely spaced sites for O-linked modification. Activation by PHA-E could be mediated by the simultaneous engagement of closely spaced heterogeneous O-linked glycans. Thus, biochemical binding assays, such as glycan arrays using a single purified O-glycan, are unlikely to reveal the diversity that is allowed in biological systems such as the one described here.

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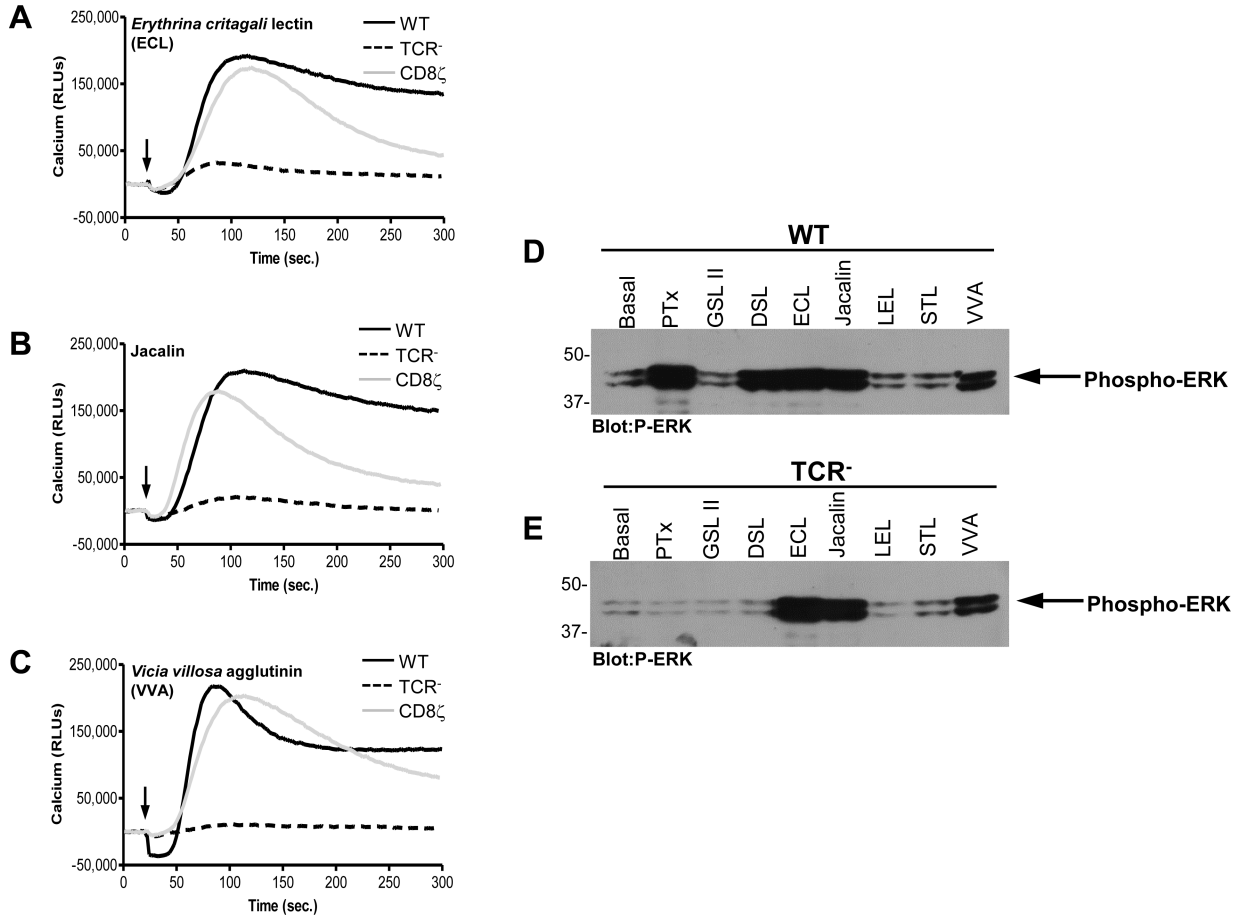

Figure 9. Calcium and ERK MAPK signaling induced by lectins ECL, VVA, and Jacalin. WT, TCR$^-$, or CD8ζ Jurkat cells were loaded with the Fluo-4 NW calcium indicator dye and stimulated with 2 μg/mL ECL (A), VVA (B), or Jacalin (C). The time at which ligand is added is marked with a black arrow. Data were collected on a Molecular Dynamics FlexStation. WT (D) or TCR$^-$ (E) Jurkat T cells were left unstimulated or stimulated with the indicated lectin (2 μg/mL) for 15 min. Whole cell extracts were analyzed by Western blotting for activated ERK with phospho-specific antibodies. Representative data are shown for each treatment.

Another deviation from predicted results is the failure of PNA to activate the CD8ζ chimera. PNA is reported to recognize Galβ1−3GalNAc, also called Thomsen-Friedenreich antigen (T-antigen),[65] which is a major O-glycan structure found on human T cells (Table S3 of the Supporting Information). However, the inability of PNA to mediate cellular aggregation suggests Jurkat cells lack PNA receptor expression. In contrast, another lectin, Jacalin, which also binds the T-antigen, can promote signaling (Table 1), suggesting that T-antigen is expressed on Jurkat cells. An explanation for these seemingly contradictory results is that binding of PNA but not Jacalin is inhibited when the T-antigen is further modified by sialylation,[66] suggesting that the majority of T-antigen present on Jurkat cells is sialylated.[67] This is further supported by the observation that tumor cells, like the Jurkat cell line, express an increased level of sialylation compared to that of normal cells.[67,68]

It is also intriguing that some lectin−receptor complexes promoted either Ca^{2+} or ERK signaling, but not both (Table 1). PHA-L and DSL activated CD8ζ leading to ERK, but not Ca^{2+}, signaling. In contrast, GSL II and LEL activated TCR leading to Ca^{2+}, but not ERK, signaling. The ability to activate either ERK or Ca^{2+} but not both could be due to quantitative or qualitative differences in receptor activation, although it is difficult to imagine how quantitative differences could lead to weakened Ca^{2+} signaling in one case and weakened ERK signaling in another. Activation of the TCR promotes receptor translocation and clustering into signaling regions called immunological synapses or supramolecular activation clusters (SMACs).[69,70] It is likely that the spatial orientation of the receptors within the SMAC can drastically affect the ability to support and sustain signaling activity. The complex and dynamic structure of the immunologic synapse makes it very difficult to study. Lectins capable of promoting the assembly of TCR- or CD8ζ-mediated signaling complexes with incomplete functional capacity (e.g., Ca^{2+} but not ERK) are very useful tools for studying the dynamic assembly of intermediates involved in the TCR signaling process.

Finally, we found that three lectins, ECL, JAC, and VVA, promoted ERK signaling, but not Ca^{2+} signaling, in cells lacking the TCR or the CD8ζ chimera (Table 1 and Figure 9). These results suggest that these lectins are capable of activating T cells by a TCR-independent signaling pathway, which has yet to be identified.

In summary, we have developed a model system for examining the ability of PTxB and other lectins to activate canonical CD3 signaling via either N-linked or O-linked glycans. Our results confirm the hypothesis that biological responses to lectins cannot be predicted simply on the basis of binding preferences determined from glycan array binding

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studies. Furthermore, the ability to cross-link a given receptor is not the sole prerequisite for driving signaling, because different lectins elicit quantitative and qualitative differences in downstream responses. This model system will be useful for probing the specific details involved in receptor activation, including defining the importance of microdomains and membrane substructures critical for recruitment of various signaling molecules to activated receptors.

■ ASSOCIATED CONTENT

Supporting Information

Glycosylation profiles for the TCR and CD8 and links to Center for Glycomics lectin−glycan binding results (Tables S1−S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ConA, concanavalin A; DBA, *D. bitflorus* agglutinin; DSL, *Da. stramonium* lectin; ECL, *E. cristagalli* lectin; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcNAc, *N*-acetylglucosamine; GSL II, *G. simplicifolia* lectin II; ITAM, immunoreceptor tyrosine-based activation motif; JAC, Jacalin; LCA, *L. culinaris* agglutinin; LEL, *Ly. esculentum* lectin; Man, mannose; Neu5Ac, *N*-acetylneuraminic acid; PHA, phytohemagluttinin; PHA-L, phytohemagluttinin leucoagglutinin; PHA-E, phytohemagluttinin erythroagglutinin; PNA, peanut agglutinin; PSA, *P. sativum* agglutinin; PTx, pertussis toxin; PTxB, pertussis toxin B subunit; RCA, *R. communis* agglutinin; SBA, soybean agglutinin; SJA, *S. japonica* agglutinin; sWGA, succinylated wheat germ agglutinin; TCR, T cell receptor; UEA-I, *U. europaetus* agglutinin; VVA, *V. villosa* agglutinin; WGA, wheat germ agglutinin; WT, wild-type.

■ REFERENCES

(1) Edelman, G. M., Cunningham, B. A., Reeke, G. N., Becker, J. W., Waxdal, M. J., and Wang, J. L. (1972) The covalent and three-dimensional structure of concanavalin A. *Proc. Natl. Acad. Sci. U.S.A. 69*, 2580−2584.

(2) Hardman, K. D., and Ainsworth, C. F. (1972) Structure of concanavalin A at 2.4-Å resolution. *Biochemistry 11*, 4910−4919.

(3) Hamelryck, T. W., Dao-Thi, M.-H., Poortmans, F., Chrispeels, M. J., Wyns, L., and Loris, R. (1996) The crystallographic structure of phytohemagglutinin-L. *J. Biol. Chem. 271*, 20479−20485.

(4) Nowell, P. (1960) Phytohemagglutinin: An initiator of mitosis in culture of animal and human leukocytes. *Cancer Res. 20*, 462−466.

(5) Hammarström, S., Hammarström, M. L., Sundblad, G., Arnarp, J., and Lönngren, J. (1982) Mitogenic leukoagglutinin from *Phaseolus vulgaris* binds to a pentasaccharide unit in N-acetyllactosamine-type glycoprotein glycans. *Proc. Natl. Acad. Sci. U.S.A. 79*, 1611−1615.

(6) Irimura, T., Kawaguchi, T., Terao, T., and Osawa, T. (1975) Carbohydrate-binding specificity of the so-called galactose-specific phytohemagglutinins. *Carbohydr. Res. 39*, 317−327.

(7) Wright, C. S. (1977) The crystal structure of wheat germ agglutinin at 2.2 Å resolution. *J. Mol. Biol. 111*, 439−457.

(8) Monsigny, M., Sene, C., Obrenovitch, A., Roche, A. C., Delmotte, F., and Boschetti, E. (1979) Properties of succinylated wheat-germ agglutinin. *Eur. J. Biochem. 98*, 39−45.

(9) Monsigny, M., Roche, A. C., Sene, C., Maget-Dana, R., and Delmotte, F. (1980) Sugar-lectin interactions: How does wheat-germ agglutinin bind sialoglycoconjugates? *Eur. J. Biochem. 104*, 147−153.

(10) Katada, T., Tamura, M., and Ui, M. (1983) The A protomer of islet-activating protein, pertussis toxin, as an active peptide catalyzing ADP-ribosylation of a membrane protein. *Arch. Biochem. Biophys. 224*, 290−298.

(11) Stein, P. E., Boodhoo, A., Armstrong, G. D., Heerze, L. D., Cockle, S. A., Klein, M. H., and Read, R. J. (1994) Structure of a pertussis toxin-sugar complex as a model for receptor binding. *Nat. Struct. Biol. 1*, 591−596.

(12) Rozdzinski, E., Burnette, W. N., Jones, T., Mar, V., and Tuomanen, E. (1993) Prokaryotic peptides that block leukocyte adherence to selectins. *J. Exp. Med. 178*, 917−924.

(13) Capiau, C., Petre, J., Van Damme, J., Puype, M., and Vandekerckhove, J. (1986) Protein-chemical analysis of pertussis toxin reveals homology between the subunits S2 and S3, between S1 and the A chains of enterotoxins of *Vibrio cholerae* and *Escherichia coli* and identifies S2 as the haptoglobin-binding subunit. *FEBS Lett. 204*, 336−340.

(14) Sekura, R. D., and Zhang, Y. (1985) Pertussis toxin: Structural elements involved in the interaction with cells. In *Pertussis Toxin* (Sekura, R. D., Moss, J., and Vaughan, M., Eds.) pp 45−64. Academic Press, Inc., Orlando, FL.

(15) Francotte, M., Locht, C., Feron, C., Capiau, C., and de Wilde, M. (1989) Monoclonal antibodies specific for pertussis toxin subunits and identification of the haptoglobin-binding site. In *Vaccines* (Lerner, R. A., Ginsberg, H., Chanock, R. M., and Brown, F., Eds.) pp 243−247. Cold Spring Harbor Laboratory Press, Plainview, NY.

(16) Loosmore, S., Zealey, G., Cockle, S., Boux, H., Chong, P., Yacoob, R., and Klein, M. (1993) Characterization of pertussis toxin analogs containing mutations in B-oligomer subunits. *Infect. Immun. 61*, 2316−2324.

(17) Schmidt, W., and Schmidt, M. A. (1989) Mapping of linear B-cell epitopes of the S2 subunit of pertussis toxin. *Infect. Immun. 57*, 438−445.
(18) Schmidt, M. A., and Schmidt, W. (1989) Inhibition of pertussis toxin binding to model receptors by antipeptide antibodies directed at an antigenic domain of the S2 subunit. *Infect. Immun. 57*, 3828−3833.
(19) Schmidt, M. A., Raupach, B., Szulczynski, M., and Marzillier, J. (1991) Identification of linear B-cell determinants of pertussis toxin associated with the receptor recognition site of the S3 subunit. *Infect. Immun. 59*, 1402−1408.
(20) Lobet, Y., Feron, C., Dequesne, G., Simoen, E., Hauser, P., and Locht, C. (1993) Site-specific alterations in the B oligomer that affect receptor-binding activities and mitogenicity of pertussis toxin. *J. Exp. Med. 177*, 79−87.
(21) Sandros, J., Rozdzinski, E., Zheng, J., Cowburn, D., and Tuomanen, E. (1994) Lectin domains in the toxin of *Bordetella pertussis*: Selectin mimicry linked to microbial pathogenesis. *Glycoconjugate J. 11*, 501−506.
(22) Saukkonen, K., Burnette, W. N., Mar, V. L., Masure, H. R., and Tuomanen, E. I. (1992) Pertussis toxin has eukaryotic-like carbohydrate recognition domains. *Proc. Natl. Acad. Sci. U.S.A. 89*, 118−122.
(23) Millen, S. H., Lewallen, D. M., Herr, A. B., Iyer, S. S., and Weiss, A. A. (2010) Identification and characterization of the carbohydrate ligands recognized by pertussis toxin via a glycan microarray and surface plasmon resonance. *Biochemistry 49*, 5954−5967.
(24) Schneider, O. D., Weiss, A. A., and Miller, W. E. (2007) Pertussis toxin utilizes proximal components of the T-cell receptor complex to initiate signal transduction events in T cells. *Infect. Immun. 75*, 4040−4049.
(25) Denkinger, C. M., Denkinger, M. D., and Forsthuber, T. G. (2007) Pertussis toxin-induced cytokine differentiation and clonal expansion of T cells is mediated predominantly via costimulation. *Cell. Immunol. 246*, 46−54.
(26) Garcia, J. G., Wang, P., Liu, F., Hershenson, M. B., Borbiev, T., and Verin, A. D. (2001) Pertussis toxin directly activates endothelial cell p42/p44 MAP kinases via a novel signaling pathway. *Am. J. Physiol. 280*, C1233−C1241.
(27) Gray, L. S., Huber, K. S., Gray, M. C., Hewlett, E. L., and Engelhard, V. H. (1989) Pertussis toxin effects on T lymphocytes are mediated through CD3 and not by pertussis toxin catalyzed modification of a G protein. *J. Immunol. 142*, 1631−1638.
(28) Hou, W., Wu, Y., Sun, S., Shi, M., Sun, Y., Yang, C., Pei, G., Gu, Y., Zhong, C., and Sun, B. (2003) Pertussis toxin enhances Th1 responses by stimulation of dendritic cells. *J. Immunol. 170*, 1728−1736.
(29) Li, H., and Wong, W. S. (2001) Pertussis toxin activates tyrosine kinase signaling cascade in myelomonocytic cells: A mechanism for cell adhesion. *Biochem. Biophys. Res. Commun. 283*, 1077−1082.
(30) Morse, J. H., Kong, A. S., Lindenbaum, J., and Morse, S. I. (1977) The mitogenic effect of the lymphocytosis promoting factor from *Bordetella pertussis* on human lymphocytes. *J. Clin. Invest. 60*, 683−692.
(31) Racke, M. K., Hu, W., and Lovett-Racke, A. E. (2005) PTX cruiser: Driving autoimmunity via TLR4. *Trends Immunol. 26*, 289−291.
(32) Rosoff, P. M., and Mohan, C. (1992) Unidirectional, heterologous desensitization of the pertussis toxin receptor by the CD3/TCR complex. *J. Immunol. 149*, 3191−3199.
(33) Sindt, K. A., Hewlett, E. L., Redpath, G. T., Rappuoli, R., Gray, L. S., and Vandenberg, S. R. (1994) Pertussis toxin activates platelets through an interaction with platelet glycoprotein Ib. *Infect. Immun. 62*, 3108−3114.
(34) Meng, J., Parroche, P., Golenbock, D. T., and McKnight, C. J. (2008) The differential impact of disulfide bonds and N-linked glycosylation on the stability and function of CD14. *J. Biol. Chem. 283*, 3376−3384.
(35) Rudd, P. M., Elliott, T., Cresswell, P., Wilson, I. A., and Dwek, R. A. (2001) Glycosylation and the immune system. *Science 291*, 2370−2376.
(36) Saitoh, S.-I., and Miyake, K. (2006) Mechanism regulating cell surface expression and activation of Toll-like receptor 4. *Chem. Rec. 6*, 311−319.
(37) Hausman, S. Z., and Burns, D. L. (1993) Binding of pertussis toxin to lipid vesicles containing glycolipids. *Infect. Immun. 61*, 335−337.
(38) Hausman, S. Z., and Burns, D. L. (1992) Interaction of pertussis toxin with cells and model membranes. *J. Biol. Chem. 267*, 13735−13739.
(39) Hausman, S. Z., Burns, D. L., Sickler, V. C., and Manclark, C. R. (1989) Immune response to dimeric subunits of the pertussis toxin B oligomer. *Infect. Immun. 57*, 1760−1764.
(40) Heerze, L. D., Chong, P. C., and Armstrong, G. D. (1992) Investigation of the lectin-like binding domains in pertussis toxin using synthetic peptide sequences. Identification of a sialic acid binding site in the S2 subunit of the toxin. *J. Biol. Chem. 267*, 25810−25815.
(41) Spangler, B. D., Heerze, L. D., Clark, C. G., and Armstrong, G. D. (1993) Hydrophobic binding of pertussis toxin is enhanced by oligosaccharide receptors. *Arch. Biochem. Biophys. 305*, 153−158.
(42) Witvliet, M. H., Burns, D. L., Brennan, M. J., Poolman, J. T., and Manclark, C. R. (1989) Binding of pertussis toxin to eucaryotic cells and glycoproteins. *Infect. Immun. 57*, 3324−3330.
(43) Rosoff, P. M., Walker, R., and Winberry, L. (1987) Pertussis toxin triggers rapid second messenger production in human T lymphocytes. *J. Immunol. 139*, 2419−2423.
(44) Tonon, S., Badran, B., Benghiat, F. S., Goriely, S., Flamand, V., Willard-Gallo, K., Willems, F., Goldman, M., and De Wit, D. (2006) Pertussis toxin activates adult and neonatal naive human CD4+ T lymphocytes. *Eur. J. Immunol. 36*, 1794−1804.
(45) Irving, B. A., and Weiss, A. (1991) The cytoplasmic domain of the T cell receptor ζ chain is sufficient to couple to receptor-associated signal transduction pathways. *Cell 64*, 891−901.
(46) Irving, B. A., Chan, A. C., and Weiss, A. (1993) Functional characterization of a signal transducing motif present in the T cell antigen receptor ζ chain. *J. Exp. Med. 177*, 1093−1103.
(47) Weiss, A., and Stobo, J. D. (1984) Requirement for the coexpression of T3 and the T cell antigen receptor on a malignant human T cell line. *J. Exp. Med. 160*, 1284−1299.
(48) Juang, Y.-T., Wang, Y., Solomou, E. E., Li, Y., Mawrin, C., Tenbrock, K., Kyttaris, V. C., and Tsokos, G. C. (2005) Systemic lupus erythematosus serum IgG increases CREM binding to the IL-2 promoter and suppresses IL-2 production through CaMKIV. *J. Clin. Invest. 115*, 996−1005.
(49) Hewlett, E. L., Sauer, K. T., Myers, G. A., Cowell, J. L., and Guerrant, R. L. (1983) Induction of a novel morphological response in Chinese hamster ovary cells by pertussis toxin. *Infect. Immun. 40*, 1198−1203.
(50) Finco, T. S., Kadlecek, T., Zhang, W., Samelson, L. E., and Weiss, A. (1998) LAT is required for TCR-mediated activation of PLCγ1 and the Ras pathway. *Immunity 9*, 617−626.
(51) Nunes, J., Klasen, S., Franco, M. D., Lipcey, C., Mawas, C., Bagnasco, M., and Olive, D. (1993) Signalling through CD28 T-cell activation pathway involves an inositol phospholipid-specific phospholipase C activity. *Biochem. J. 293* (Part 3), 835−842.
(52) Pear, W. S., Miller, J. P., Xu, L., Pui, J. C., Soffer, B., Quackenbush, R. C., Pendergast, A. M., Bronson, R., Aster, J. C., Scott, M. L., and Baltimore, D. (1998) Efficient and rapid induction of a chronic myelogenous leukemia-like myeloproliferative disease in mice receiving P210 bcr/abl-transduced bone marrow. *Blood 92*, 3780−3792.
(53) Merry, A. H., Gilbert, R. J. C., Shore, D. A., Royle, L., Miroshnychenko, O., Vuong, M., Wormald, M. R., Harvey, D. J., Dwek, R. A., Classon, B. J., Rudd, P. M., and Davis, S. J. (2003) O-Glycan sialylation and the structure of the stalk-like region of the T cell co-receptor CD8. *J. Biol. Chem. 278*, 27119−27128.

(54) Julius, M., Maroun, C. R., and Haughn, L. (1993) Distinct roles for CD4 and CD8 as co-receptors in antigen receptor signalling. *Immunol. Today 14*, 177−183.
(55) Chan, A. C., Irving, B. A., Fraser, J. D., and Weiss, A. (1991) The ζ chain is associated with a tyrosine kinase and upon T-cell antigen receptor stimulation associates with ZAP-70, a 70-kDa tyrosine phosphoprotein. *Proc. Natl. Acad. Sci. U.S.A. 88*, 9166−9170.
(56) Chan, A. C., Irving, B. A., and Weiss, A. (1992) New insights into T-cell antigen receptor structure and signal transduction. *Curr. Opin. Immunol. 4*, 246−251.
(57) Rudd, P. M., Wormald, M. R., Stanfield, R. L., Huang, M., Mattsson, N., Speir, J. A., DiGennaro, J. A., Fetrow, J. S., Dwek, R. A., and Wilson, I. A. (1999) Roles for glycosylation of cell surface receptors involved in cellular immune recognition. *J. Mol. Biol. 293*, 351−366.
(58) Gupta, R., and Brunak, S. (2002) Prediction of glycosylation across the human proteome and the correlation to protein function. *Pac. Symp. Biocomput. 2002,*, 310−322.
(59) Julenius, K., Mølgaard, A., Gupta, R., and Brunak, S. (2005) Prediction, conservation analysis, and structural characterization of mammalian mucin-type O-glycosylation sites. *Glycobiology 15*, 153−164.
(60) Iwashima, M., Irving, B. A., van Oers, N. S., Chan, A. C., and Weiss, A. (1994) Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases. *Science 263*, 1136−1139.
(61) Straus, D. B., Chan, A. C., Patai, B., and Weiss, A. (1996) SH2 domain function is essential for the role of the Lck tyrosine kinase in T cell receptor signal transduction. *J. Biol. Chem. 271*, 9976−9981.
(62) Straus, D. B., and Weiss, A. (1993) The CD3 chains of the T cell antigen receptor associate with the ZAP-70 tyrosine kinase and are tyrosine phosphorylated after receptor stimulation. *J. Exp. Med. 178*, 1523−1530.
(63) Chilson, O. P., Boylston, A. W., and Crumpton, M. J. (1984) *Phaseolus vulgaris* phytohaemagglutinin (PHA) binds to the human T lymphocyte antigen receptor. *EMBO J. 3*, 3239−3245.
(64) Narasimhan, S., Freed, J. C., and Schachter, H. (1986) The effect of a "bisecting" N-acetylglucosaminyl group on the binding of biantennary, complex oligosaccharides to concanavalin A, *Phaseolus vulgaris* erythroagglutinin (E-PHA), and *Ricinus communis* agglutinin (RCA-120) immobilized on agarose. *Carbohydr. Res. 149*, 65−83.
(65) Skutelsky, E., Lotan, R., Sharon, N., and Danon, D. (1977) Distribution of the T-antigen on erythroid cell surfaces. Studies with peanut agglutinin, an anti-T specific lectin. *Biochim. Biophys. Acta 467*, 165−174.
(66) Novogrodsky, A., Lotan, R., Ravid, A., and Sharon, N. (1975) Peanut agglutinin, a new mitogen that binds to galactosyl sites exposed after neuraminidase treatment. *J. Immunol. 115*, 1243−1248.
(67) Yogeeswaran, G., and Salk, P. L. (1981) Metastatic potential is positively correlated with cell surface sialylation of cultured murine tumor cell lines. *Science 212*, 1514−1516.
(68) Miyagi, T., Wada, T., Yamaguchi, K., and Hata, K. (2004) Sialidase and malignancy: A minireview. *Glycoconjugate J. 20*, 189−198.
(69) Schamel, W. W. A., and Reth, M. (2008) Clustering models. *Adv. Exp. Med. Biol. 640*, 64−73.
(70) Jacobelli, J., Andres, P. G., Boisvert, J., and Krummel, M. F. (2004) New views of the immunological synapse: Variations in assembly and function. *Curr. Opin. Immunol. 16*, 345−352.